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(21) International Application Number: PCT/GB98/02517 (22) International Filing Date: 21 August 1998 (21.08.98) (30) Priority Data: <table border="0"> <tr> <td>9717913.9</td> <td>22 August 1997 (22.08.97)</td> <td>GB</td> </tr> <tr> <td>9812822.6</td> <td>12 June 1998 (12.06.98)</td> <td>GB</td> </tr> </table> (71) Applicant (for all designated States except US): BIOTECHNOLOGY AND BIOLOGICAL SCIENCES RESEARCH COUNCIL [GB/GB]; Central Office, Polaris House, North Star Avenue, Swindon SN2 1UH (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SANG, Helen [GB/GB]; Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS (GB). FINNEGAN, David, John [GB/GB]; University of Edinburgh, Old College, South Bridge, Edinburgh EH8 9YL (GB). (74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).		9717913.9	22 August 1997 (22.08.97)	GB	9812822.6	12 June 1998 (12.06.98)	GB	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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9812822.6	12 June 1998 (12.06.98)	GB						
(54) Title: USE OF MARINER TRANSPOSAN IN THE PRODUCTION OF TRANSGENIC ANIMALS (57) Abstract <p>A method for the preparation of a transgenic animal embryo comprising the step of introducing a <i>mariner</i>-like element (MLE) containing a transgene into an animal embryo cell, optionally including the step of introduction of exogenous transposase protein, or a DNA or RNA sequence encoding a transposase. The resulting embryo may be used to generate further embryos or be allowed to develop into an animal. The invention is useful in introducing foreign DNA into selected animals.</p>								

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USE OF *MARINER* TRANSPOSAN IN THE PRODUCTION OF TRANSGENIC ANIMALS

The present invention relates to a method for introducing a transgene into an animal embryo and to the preparation of a transgenic animal therefrom.

5 Introduction of foreign DNA by microinjection into the newly fertilised egg, before the first embryonic cell cleavage, has become an established method for the production of transgenic animals. The foreign DNA is incorporated into the chromosomes of the animal and inherited as a stable additional sequence by the offspring of the founder transgenic animal.

10

The frequency at which this chromosomal integration occurs varies between species. The frequency is also influenced by the site of injection. If the DNA is introduced into one of the pronuclei of the fertilised egg, the frequency of production of transgenic animals is generally higher.

15

Transposable elements are defined sequences of DNA that can transpose to different sites in the genome of an organism. Transposable elements can be divided into several different classes, defined by the mechanism which they use to move from one genomic site to another. The ability of transposable elements has been modified to enable use of a transposable element from a particular species to be used as a vector to introduce foreign DNA into the genome of that species, e.g. the *P* element from *Drosophila melanogaster* is widely used to transform *D. melanogaster* (Rubin, G. M., & Spradling, A. C., *Science* **218** 348-353 (1982) and US-A-4670388).

20

25 A method for culturing the chick embryo from the newly fertilised egg to give hatched chicks has been developed and is described in EP-A-0295964 and in Perry, M. M. *Nature* **331** 70-72 (1988). Subsequently, a method for injecting DNA into the cytoplasm of the chick zygote, i.e. the germinal disc, was described in Sang, H. M. and Perry, M. M., *Mol. Reprod. Development* **1** 98-106 (1989) and in Perry *et al*
30 *Roux's Archive of Developmental Biology* **200** 312-319 (1991). The use of these

techniques in the production of a transgenic bird was reported in Love *et al Bio/Technology* 12 60-63 (1994). However, continued use of this procedure for the production of transgenic birds has shown that the frequency at which transgenic birds can be obtained, defined by the incorporation of foreign DNA which is transmitted to their offspring, is low: 3 germline transgenic birds from a total of 254 live chicks were transgenic.

The efficiency of this method is therefore relatively poor with only 1% or less of chicks hatched after DNA injection have incorporated the injected DNA into their genome. Additionally, from each of these transgenic birds only a single transgenic line has been obtained, i.e. the transgenic offspring contain a few copies of the foreign DNA at a single chromosome site.

Injection of DNA constructs into the cytoplasm is also very inefficient in mammals when compared to pronuclear injection (Brinster *et al Proc. Nat'l. Acad. Sci. USA* 82 4438-4442 (1985)). Also most transgenes produced by pronuclear injection consist of an array of multiple copies of the injected DNA construct. The organisation of these arrays can have negative effects on expression of the transgene, e.g. reduce the level of expression or affect the tissue-specificity of expression.

Transgenic birds have also been produced using infection of retroviral vectors (Bosselman *et al Science* 243 533-535 (1989)). However, the use of retroviral vectors has several disadvantages. The risk of recombination of viral vectors with wild type retroviruses which are widespread in poultry populations is perceived as the most serious problem. Retroviral vectors are also very complicated to work with and are restricted in their capacity to incorporate constructs greater than approximately 8 kilobases of DNA.

The transposable element *mariner* was originally discovered in the genome of *Drosophila mauritiana* but closely related elements have been discovered in a wide variety of species both vertebrate and invertebrate (Robertson, H. M., *Nature* 362 241

- (1993)). It has also been used to investigate pathogenic organisms such as *Leishmania* (Gueiros-Filho, F. J., & Beverley, S. M., *Science* 276 1716-1719 (1997) and Hartl, D. L., *Science* 276 1659-1660 (1997). However, the use of *mariner* described by Gueiros-Filho *et al* and Hartl only relate to its use as a genetic tool, i.e.
- 5 for insertional mutagenesis and not as a means for the preparation of transgenic animals by transgenesis or mutagenesis. In summary, *mariner* has not been described as being suitable for a role in the production of transgenic animals and nor is such a use contemplated in the prior art.
- 10 It has now been found surprisingly that the *mariner* element can be used as a vector in the preparation of transgenic animals.

According to a first aspect of the present invention there is provided a method for the preparation of a transgenic animal embryo comprising the step of introducing a

15 *mariner*-like element (MLE) containing a transgene into an animal embryo cell, optionally including the step of introduction of exogenous transposase protein, or a DNA or RNA sequence encoding a transposase.

The term transgenic is used in the context of the present invention to describe animals

20 which have stably incorporated a sequence of foreign DNA introduced by the *mariner*-like element (MLE) into their chromosomes such it that may passed on to successive generations of transgenic descendant animals. In such circumstances, the initial transgenic animal is known as a "founder" animal. The founder animal may have the foreign DNA or transgene incorporated in all of its cells or a sufficient

25 proportion such that its progeny stably inherit the transgene. Where the transgene is only present in a proportion of cells of the animal, the animal is referred to as a chimera. The present invention also extends to animals which incorporate the transgene stably or directly into their chromosomes and which express the transgene in their somatic cells without passing the gene onto their offspring.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line
5 has been the subject of the introduction of a *mariner*-like element (MLE). So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose germ line an exogenous DNA sequence has been added.

10

In principle, the invention is applicable to all animals, including birds, such as domestic fowl, amphibian species and fish species. In practice, however, it will be to non-human animals (warm-blooded vertebrates), especially (non-human) mammals, particularly placental mammals, and birds, particularly poultry, that the greatest
15 commercial useful applicability is presently envisaged. It is with ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs that the invention is likely to be most useful. Of the avian species, the invention has particular application to poultry, including domestic fowl *Gallus domesticus*, turkeys and guinea fowl. It should also be noted that the
20 invention is also likely to be applicable to other economically important animal species such as, for example, horses, llamas or rodents, e.g. rats, mice or rabbits.

The method of the present invention is directed towards the introduction of foreign DNA or a transgene into an animal embryo cell. The embryo cell may be at the
25 single cell stage immediately following fertilisation which is the zygote stage. However, the introduction may be into an embryo cell from a later stage of embryonic development, e.g. from a 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, or 64-cell stage embryo, or from an even later stage. A founder transgenic animal produced from such a later stage embryo may therefore be a chimera but its offspring can be
30 selected for the presence of the transgene in all cells.

The *mariner*-like element may be the transposable element *mariner* from *Drosophila mauritiana* or a closely related element from another vertebrate or invertebrate species (Robertson, H. M., *Nature* 362 241 (1993)). The *mariner*-like element may conveniently be derived from the cells of the animal whose chromosomes are to
5 altered. A nucleotide sequence which is a *mariner*-like element can be defined by its ability to act as a transposable element when introduced into a cell.

Mariner-like transposable elements are about 1,300bp long with terminal inverted repeats of about 30bp. Each *mariner*-like element encodes a polypeptide that is a
10 putative transposase and that has, on average, 34% amino acid sequence identity with the polypeptides encoded by other *mariner*-like elements. The amino acid sequences of the putative transposases of all *mariner*-like elements include a characteristic motif known as D, D34D, where "D" represents an aspartate residue. The third aspartate of this motif is followed immediately by a tyrosine residue (Robertson, H. M., *J. Insect*
15 *Physiol.* 41 99-105 (1995)).

The transgene can be contained in the *mariner*-like element at any point within the *mariner* sequence. Without being bound by theory, it is believed that approximately the final 100 bases of each end of the *mariner*-like element may be important for
20 function of the MLE and its incorporation into the chromosomes of a cell. Thus the transgene may be positioned anywhere within in the MLE except less than approximately 100 bases from each end. The transgene may also replace the central sequences of the MLE, with only the ends of the element being retained.

25 The transgene sequence contained in the *mariner*-like element may be any desired foreign gene sequence. Particularly preferred gene sequences include, but are not limited to, those gene sequences coding for proteins which are therapeutically useful, such as enzymes, hormones or other functionally active proteins, e.g. immunoglobulins, haemoglobin, myoglobin, cytochromes, etc. Other gene sequences
30 may encode proteins whose genes are absent or mutated such that the corresponding

protein is not produced or is not produced in active form, i.e. genes responsible for disease conditions such as cystic fibrosis or muscular dystrophy.

Typically, the transgene sequences will also contain promoter sequences to direct
5 expression of the transgene in a selected tissue, e.g. the mammary gland for secretion in the animals milk, in the yolk or albumen of an egg, or in the blood. Further applications, include the expression of regulatory proteins that control immune rejection such that the organs of the transgenic host animal may be used in Xenotransplantation into a recipient which is allogenic for the immune proteins being
10 expressed in the cells. Similarly, allotransplantation is also included.

In agricultural applications of the method of the present invention may be used to produce improved farm animals. The transgenes introduced into the animals may include, but are not limited to, disease resistance genes, growth enhancing genes or
15 genes which provide for improved characteristics in a particular trait or introduction of a novel trait.

The introduction of the *mariner*-like element (MLE) may conveniently be achieved by injection of the MLE into the cytoplasm or into the pronucleus of a zygote or the
20 nucleus of an animal embryo cell. Other routes of introduction, such as electroporation or liposomes may be equally effective and used in the method according to the present invention.

The MLE may be introduced in the form of a construct comprising the DNA
25 sequence of a *mariner*-like element and the desired transgene or simply the nucleotide sequence of a *mariner*-like element and the desired transgene itself may be introduced. Where a vector based method of introduction is used, the construct may be a plasmid, a cosmid or an artificial chromosome such as a Yeast Artificial Chromosome (YAC) or a Bacterial Artificial Chromosome (BAC). The constructs
30 may also contain additional regulatory sequences, if required, such as promoters or enhancers, depending of the foreign DNA being introduced. A further aspect of the

present invention is therefore a construct or a vector comprising a *mariner*-like element containing a transgene as described above. In general, the MLE will be cloned in a plasmid vector for ease of manipulation and cloning-in of transgenes. It may also be preferable to have the MLE vector in a circular molecule so that the

5 DNA will be supercoiled to facilitate transposition.

At the time of introduction of the *mariner*-like element, or shortly before or shortly thereafter, the method of the present invention may contain an additional optional step of introducing exogenous transposase protein, or a DNA or RNA sequence encoding

10 a transposase.

The *mariner*-like element and the transposase introduced into the cell may be derived from the same animal species or different species.

15 According to a second aspect of the present invention there is provided a method for the preparation of a transgenic animal embryo comprising the step of introducing a *mariner*-like element (MLE) containing a transgene into an animal adult cell, optionally including the step of introduction of exogenous transposase protein, or a DNA or RNA sequence encoding a transposase.

20 According to a third aspect of the present invention there is provided a method for the preparation of a transgenic animal embryo comprising the step of introducing a *mariner*-like element (MLE) containing a transgene into an animal foetal cell, optionally including the step of introduction of exogenous transposase protein, or a

25 DNA or RNA sequence encoding a transposase.

In any one of the methods according to the first, second and third aspects of the present invention, the resulting animal embryo may be prepared by (i) removing the nucleus of the cell following introduction of the *mariner*-like element containing the

30 transgene and its insertion into the chromosomes, and (ii) subsequently introducing the nucleus into an enucleated oocyte which is allowed to develop into an animal

embryo. There are several methods described for the preparation of an animal embryo using nuclear transfer techniques and preferred techniques include, but are not limited to those described in WO-A-9607732, WO-A-9707669 and WO-A-9707668.

5

In the method of the invention described above, a nucleus is transferred from a donor cell to a recipient cell. The use of this method is not restricted to a particular donor cell type. The donor cell may be as described in Wilmut *et al Nature* 385 810 (1997); Campbell *et al Nature* 380 64-66 (1996); or Cibelli *et al Science* 280 1256-1258
10 (1998). All cells of normal karyotype, including embryonic, foetal and adult somatic cells which can be used successfully in nuclear transfer may in principle be employed in a method according to the present invention. Foetal fibroblasts are a particularly useful class of donor cells. Generally suitable methods of nuclear transfer are described in Campbell *et al Theriogenology* 43 181 (1995), Collas *et al Mol. Reprod.*
15 *Dev.* 38 264-267 (1994), Keefer *et al Biol. Reprod.* 50 935-939 (1994), Sims *et al Proc. Nat'l. Acad. Sci. USA* 90 6143-6147 (1993), WO-A-9426884, WO-A-9424274, WO-A-9807841, WO-A-9827214, WO-A-9003432, US-A-4994384 and US-A-5057420. The invention therefore contemplates the use of an at least partially differentiated cell, including a fully differentiated cell. Donor cells may be, but do
20 not have to be, in culture and may be quiescent. Nuclear donor cells which are quiescent are cells which can be induced to enter quiescence or exist in a quiescent state *in vivo*. Cultured bovine primary fibroblasts, an embryo-derived ovine cell line (TNT4), an ovine mammary epithelial cell derived cell line (OME) from a 6 year old adult sheep, a fibroblast cell line derived from foetal ovine tissue (BLWF1) and an
25 epithelial-like cell line derived from a 9-day old sheep embryo (SEC1) are described in WO-A-9707669 and WO-A-9707668. A class of embryo-derived cell lines useful in the invention which includes the TNT4 cell line described in WO 96/07732. Cultured inner cell mass (CICM) cells are described in WO-A9737009 and WO-A-9827214 and embryonic or stem-like cell lines are described in WO-A-9807841.
30 Transgenic bovine fibroblasts for use as nuclear donors are described in Zawada *et al*

(*Nature Medicine* 4 (5) 569-574 (1998) and in Cibelli *et al* (*Science* 280 1256-1258 (1998)).

Where the donor cells are described as being quiescent, such cells may not be actively proliferating by means of the mitotic cell cycle. The use of a quiescent donor cell is
5 described in WO-A-9707669. The mitotic cell cycle has four distinct phases, G1, S, G2 and M. The beginning event in the cell cycle, called *start*, takes place in the G1 phase and has a unique function. The decision or commitment to undergo another cell cycle is made at *start*. Once a cell has passed through *start*, it passes through the remainder of the G1 phase, which is the pre-DNA synthesis phase. The second stage,
10 the S phase, is when DNA synthesis takes place. This is followed by the G2 phase, which is the period between DNA synthesis and mitosis. Mitosis itself occurs at the M phase. Quiescent cells (which include cells in which quiescence has been induced as well as those cells which are naturally quiescent, such as certain fully differentiated cells) are generally regarded as not being in any of these four phases of the cycle; they
15 are usually described as being in a G0 state, so as to indicate that they would not normally progress through the cycle. The nuclei of quiescent G0 cells have a diploid DNA content.

Cultured cells can be induced to enter the quiescent state by various methods
20 including chemical treatments, nutrient deprivation, growth inhibition or manipulation of gene expression. Presently the reduction of serum levels in the culture medium has been used successfully to induce quiescence in both ovine and bovine cell lines. In this situation, the cells exit the growth cycle during the G1 phase and arrest, as explained above, in the so-called G0 stage. Such cells can remain in
25 this state for several days (possibly longer depending upon the cell) until re-stimulated when they re-enter the growth cycle. Quiescent cells arrested in the G0 state are diploid. The G0 state is the point in the cell cycle from which cells are able to differentiate. On quiescence a number of metabolic changes have been reported and these include: monophosphorylated histones, ciliated centrioles, reduction or
30 complete cessation in all protein synthesis, increased proteolysis, decrease in transcription and increased turnover of RNA resulting in a reduction in total cell

RNA, disaggregation of polyribosomes, accumulation of inactive 80S ribosomes and chromatin condensation (reviewed Whitfield *et al.*, *Control of Animal Cell Proliferation*, 1 331-365 (1985)).

- 5 Many of these features are those which are required to occur following transfer of a nucleus to an enucleated oocyte. The fact that the G0 state is associated with cell differentiation suggests that this may provide a nuclear/chromatin structure which is more amenable to either remodelling and/or reprogramming by the recipient cell cytoplasm. In this way, by virtue of the nuclear donor cells being in the quiescent
- 10 state, the chromatin of the nuclei of the donors may be modified before embryo reconstitution or reconstruction such that the nuclei are able to direct development. This differs from all previously reported methods of nuclear transfer in that the chromatin of donor cells is modified prior to the use of the cells as nuclear donors.
- 15 The recipient cell to which the nucleus from the donor cell is transferred may be an oocyte or another suitable cell. A preferred class of recipient oocyte is described in WO-A-9707668.

- Recipient cells at a variety of different stages of development may be used, from
- 20 oocytes at metaphase I through metaphase II, to zygotes and two-cell embryos. Each has its advantages and disadvantages. The use of fertilized eggs ensures efficient activation whereas parthenogenetic activation is required with oocytes (see below). Another mechanism that may favour the use of cleavage-stage embryos in some species is the extent to which reprogramming of gene expression is required.
- 25 Transcription is initiated during the second cell cycle in the mouse and no major changes in the nature of the proteins being synthesised are revealed by two-dimensional electrophoresis until the blastocyst stage (Howlett & Bolton *J. Embryol. Exp. Morphol.* 87 175-206 (1985)). In most cases, though, the recipient cells will be oocytes.

It is preferred that the recipient be enucleate. While it has been generally assumed that enucleation of recipient oocytes in nuclear transfer procedures is essential, there is no published experimental confirmation of this judgement. The original procedure described for ungulates involved splitting the cell into two halves, one of which was
5 likely to be enucleated (Willadsen *Nature* **320** (6) 63-65 (1986)). This procedure has the disadvantage that the other unknown half will still have the metaphase apparatus and that the reduction in volume of the cytoplasm is believed to accelerate the pattern of differentiation of the new embryo (Eviskov *et al.*, *Development* **109** 322-328 (1990)).

10

More recently, different procedures have been used in attempts to remove the chromosomes with a minimum of cytoplasm. Aspiration of the first polar body and neighbouring cytoplasm was found to remove the metaphase II apparatus in 67% of sheep oocytes (Smith & Wilmut *Biol. Reprod.* **40** 1027-1035 (1989)). Only with the
15 use of DNA-specific fluorochrome (Hoechst 33342) was a method provided by which enucleation would be guaranteed with the minimum reduction in cytoplasmic volume (Tsunoda *et al.*, *J. Reprod. Fertil.* **82** 173 (1988)). In livestock species, this is probably the method of routine use at present (Prather & First *J. Reprod. Fertil. Suppl.* **41** 125 (1990), Westhusin *et al.*, *Biol. Reprod. (Suppl.)* **42** 176 (1990)).

20

There have been very few reports of non-invasive approaches to enucleation in mammals, whereas in amphibians, irradiation with ultraviolet light is used as a routine procedure (Gurdon *Q. J. Microsc. Soc.* **101** 299-311 (1960)). There are no detailed reports of the use of this approach in mammals, although during the use of
25 DNA-specific fluorochrome it was noted that exposure of mouse oocytes to ultraviolet light for more than 30 seconds reduced the developmental potential of the cell (Tsunoda *et al.*, *J. Reprod. Fertil.* **82** 173 (1988)).

According to a fourth aspect of the present invention there is provided a method for
30 the preparation of an animal, the method comprising the steps of:

- (a) preparing an embryo according to any of the preceding aspects of the present invention;
- (b) causing an animal to develop to term from the embryo; and
- (c) optionally breeding from the animal so formed.

5

The animal embryo prepared in accordance with this aspect of the present invention may be further manipulated prior to full development of the embryo. This may include the introduction of additional genetic material or to assay the embryo for particular genetic characteristics or the presence or absence of a gene. It is also
10 possible that more than one animal can be derived from the embryo where the cells of the embryo are used to prepare more than one embryo allowed to develop to term.

The present invention therefore also extends to an animal prepared by a method according to the fourth aspect of the invention.

15

According to a fifth aspect of the present invention there is provided the use of a *mariner*-like element in the therapy of a disease condition caused by the absence of a gene or the mutation of a gene. This aspect of the invention also extends to the use of a *mariner*-like element in the preparation of an agent for the prophylaxis or treatment
20 of a disease caused by the absence of a gene or the mutation of a gene. Such methods of treatment may comprise the introduction of a *mariner*-like element containing a transgene into an animal cell. Where transposase protein, or a DNA or RNA sequence encoding transposase is also to be introduced, this step may be simultaneous, sequential or separate to the introduction of the MLE.

25

The present invention therefore also extends to the preparation of an embryo according to any one of the preceding aspects of the invention in which the cells of the embryo are used in the treatment of a disease condition associated with the absence of a gene or the mutation of a gene. Such cells may also be used to treat
30 disease conditions in which the patient's cells are no longer active or effective, especially neurological or hormonal disorders.

Preferred features for the second and subsequent features are as for the first aspect *mutatis mutandis*.

5 The present invention will now be described with reference to the accompanying Examples and drawings which are included for the purposes of example only and are not to be construed as being limiting on the present invention. In the following Examples, reference is made to a number of drawings in which:

10 FIGURE 1 shows PCR analysis of DNA extracted from embryos and chicks that survived for at least 12 days of incubation after injection of the *mariner*-containing plasmid *pMos1*. FIGURE 1(a) shows a diagram of *pMos1*, indicating sequences identified by PCR and unique restriction sites. FIGURE 1(b) shows a graphical presentation of the results given in Table
15 1. The estimated copy number per genome equivalent of a lysozyme gene construct, injected in a previous series of experiments (n=186), is compared to the estimated copy number of *mariner* sequence. The results of injecting *pMos1* with and without the addition of recombinant-derived transposase protein are compared.

20 FIGURE 2 shows a Southern blot analysis of genomic DNA isolated from individual G₁ transgenic chicks, hybridised with a *mariner* probe. FIGURE 2(a) shows BamHI/HindIII digests of individual chicks which each have a novel pattern of *mariner*-hybridising fragments (lanes 1 to 7), and of the
25 parent G₀ cockerel (lane 8). A control digest of non-transgenic chick DNA was run in lane 9. FIGURE 2(b) shows EcoRI digests of samples from the same birds as in FIGURE 2(a). The Band arrowed in lane 2 is the EcoRI fragment cloned in pZAP13 (see FIGURE 3).

30 FIGURE 3 shows the characterisation of a single integrated *mariner* element. FIGURE 3(a) shows the Southern blot of genomic DNA from

individual G₁ chicks digested with BamHI and HindIII (from FIGURE 2(a)) was stripped and reprobed with the EcoRI insert from pZAP13. Hybridisation to a range of restriction fragments can be seen in all the samples, including the negative control (lane 9). FIGURE 3(b) shows a comparison of the sequence across the left and right ends of the *mariner* element in pMos1 and pZAP13.

FIGURE 4 shows PCR analysis for present of the Tet^R gene in DNA from embryos and chicks that survived for at least 12 days of incubation after introduction of pMos1Tet. The copy number of the Tet^R gene was estimated as described in the "Materials and Methods" and the results of co-injection of transposase protein compared with injection of plasmid alone.

Example 1: Preparation of Transposase

- 15 The *mariner* transposase used in the following experiments was purified from *E. coli* strain BL21 DE3 (Studier *et al Methods in Enzymology* 185 60-89 (1991)) carrying the plasmid pBCPMos1. This was derived from the expression vector pBCP368 (Velterop *et al Gene* 153 63-65 (1995)). The complete coding sequence of *mariner* transposase from the element *Mos1* was inserted at the *NdeI* site of pBCPMos1.
- 20 These cells were grown in Luria broth (LB) in an orbital shaker (200rpm, 37°C) to an OD₅₅₀ of 0.8 when they were induced for two hours by the addition of IPTG to 0.5mM. Following induction, the cells were harvested and stored at -20°C until required. The cells in the pellet from a 1 litre culture were resuspended in 5ml of 50mM Tris-HCl (pH7.5), 10% glycerol, 2mM MgCl₂, 1mM DTT. Lysozyme was
- 25 added to a concentration of 0.1mg/ml and the cells incubated for five minutes at room temperature. They were then lysed by the addition of 10ml of detergent buffer containing 25mM Tris-HCl (pH7.5), 4mM EDTA, 0.2M NaCl, 1% deoxycholate, 1% NP40, 1mM DTT and incubated at room temperature for a further 15 minutes. MgCl₂ was added to a final concentration of 10mM with 100µl of a 2000 units/ml
- 30 DNaseI solution. The extract was pipetted up and down a few times until the

viscosity decreased and was then left at room temperature for 10 minutes. The whole cell extract was then centrifuged at 20,000g for 30 minutes. The pellet was washed three times in 0.5% NP40 (v/v), 1mM EDTA and followed by one wash in 6M urea before being finally being resuspended in 1ml of 25mM Tris-HCl (pH7.5), 6M
5 guanidine hydrochloride, 5mM DTT. After centrifugation at 13,000g for 10 minutes, the supernatant was diluted one hundred-fold into 25mM Tris-HCl (pH7.5), 8M urea, 5mM DTT, 10% glycerol buffer and loaded onto a 2ml fast flow CM Sepharose column (Sigma) pre-equilibrated with the same buffer supplemented with 50mM NaCl.

10

Under these conditions, denatured *mariner* transposase bound to the column. Protein was renatured on the column by passing a 200ml linear gradient of 8-0M urea at a rate of 1ml/min. Following renaturation, bound protein was eluted with a 20ml linear NaCl gradient of 50mM-1.0M in buffer A (20mM Tris-HCl pH7.5, 1mM DTT, 10%
15 glycerol). Fractions containing *mariner* transposase were identified by SDS-PAGE and further concentrated by spinning through a Centricon (Amicon) column (30K molecular weight cut-off). The protein was stored frozen at a concentration of about 0.25-0.5mg/ml.

20 Example 2: Injection of *mariner* into chicken embryos

Plasmid *Mos1* containing the *mariner* element (Medhora *et al Genetics* 128 311-318 (1991)) was injected into chicken embryos as described by Sang & Perry (*Mol. Reprod. Dev.* 1 98-106 (1989)) at a concentration of 25µg/ml together with purified *mariner* transposase at a concentration of 0.05-0.005mg/ml in a buffer containing
25 100mM NaCl, 25mM HEPES pH7.7, 2mM dithiothreitol, 5% (v/v) glycerol, 25µg/ml bovine serum albumin, with or without 5mM Manganese acetate. The injected embryos were cultured as described by Perry (*Nature* 331 70-72 (1988)). Hatched chicks which had cells containing *mariner* sequences were identified by carrying out polymerase chain reactions (PCR) with primers specific to *mariner* and DNA
30 prepared from the chorioallantoic membrane of the chicks at hatch. Embryos which

died during the experiments but that survived for at least 12 days after injection were also analysed for the presence of *mariner* sequences.

Examples 3 to 8: Materials and Methods:

5 Plasmid Constructs and Preparation of Transposase protein

The plasmid pMos1Tet was constructed by insertion of the tetracyclin resistance gene into the unique Sall site present in the open reading frame of *mariner* in pMos1. The Tet^R gene was obtained by digestion of pBR322 with AvaI and EcoRI, pMos1 was linearised with Sall and the two fragments ligated after
10 treatment with Klenow polymerase to fill in the ends. The expression and preparation of recombinant-derived *mariner* transposase will be described in detail elsewhere (A. Dawson and D. Finnegan, in preparation). Briefly: the *mariner* transposase gene from pMos1 was inserted into the expression vector pBCP368 (Velterop *et al Gene* **153** 63-65 (1995)) to generate the construct pBCPMos1. This
15 construct was transferred into *E. coli* strain DH5 α and the cells harvested after induction of protein expression. The transposase protein was recovered as an insoluble precipitate, solubilised and bound to fast-flow CM sepharose column (Sigma). The protein was renatured in 8M urea and the activity measured in an *in vitro* transposition assay.

20

Microinjection and Chick Embryo Culture

Chick embryo culture was essentially as described (Perry *Nature* **331** 70-72 (1988)) with modifications noted in (Love *et al Bio/Technology* **12** 60-63 (1994)). Between 1 and 2nL of uncut plasmid, at a concentration of 25 μ g/ml, was injected
25 into the germinal disc of zygotes following established procedures (Love *et al Bio/Technology* **12** 60-63 (1994)). The DNA was diluted in transposition buffer (100mM NaCl, 25mM HEPES pH7.9, 2mM dithiothreitol, 50mM manganese acetate, 25 μ g/ml BSA, 5% glycerol) and transposase protein added when required to a concentration of 15ng/ml.

30

PCR Analysis

Tissue samples (chorioallantoic membrane, liver and gonads) were dissected from embryos which died in culture after more than 12 days of incubation and DNA extracted using Puregene (Flowgen) genomic DNA purification kit. Genomic DNA samples were obtained from chorioallantoic membrane at hatch of surviving chicks, blood samples from older birds and semen from the mature cockerel. PCR analysis was carried out on 0.5-1µg DNA samples for the presence of the *mariner* element and pBluescript (*pMos1* experiments) or for Tet^R gene and the vector chloramphenicol (CAT) resistance gene (*pMos1Tet* experiments). Control PCR reactions, to estimate copy number, were carried out in parallel on 1µg aliquots of chicken genomic DNA with *pMos1* or *pMos1Tet* DNA added in quantities equivalent to that of a single copy gene (1X) a 10-fold dilution (0.1X) and a 100-fold dilution (0.01X) as described previously (Love *et al Bio/Technology* **12** 60-63 (1994)). The primers used were:

15

(i) *mariner*

+ 5'-TCAGAAGGTCGGTAGATGGG
- 5'-AAATGACACCGCTCTGATCC

20

(ii) pBluescript

+ 5'-GCAGAGCGAGGTATGTAGGC
- 5'-AGCCCTCCCGTATCGTAGTT

25

(iii) Tet^R

+ 5'-CTTGAGAGCCTTCAACCCAG
- 5'-TTTGCGCATTACAGTTCTC

30

(iv) CAT

+ 5'-AAAATGAGACGTTGATCGGC
- 5'-AGGTTTTACCGTAACACGC

PCR products were analysed on 1.5% agarose gels and the copy number of the construct sequences estimated by comparison with the control reactions.

Southern Transfer Analysis and Isolation of Integrated *Mariner* Element

- 5 DNA from G₁ chicks, identified as transgenic by PCR, was digested with BamHI plus HindIII and EcoRI, electrophoresed through 1% agarose gel and transferred to HybondN (Amersham). A *mariner*-specific probe was generated by PCR using primers close to the ends of the element and labelled by random-priming (Rediprime, Amersham). A 0.1µg aliquot of EcoRI digested DNA from G₁chick
- 10 13 was ligated to 1µg of Lambda Zap II EcoRI-cut arms (Stratagene) and packaged with Gigapack Gold (Stratagene). Approximately 250,000 plaques were plated and screened with a *mariner*-specific probe. One positive plaque was identified, purified and the insert rescued as a plasmid following the Stratagene protocol. This clone, pZap13, was digested with EcoRI, the insert size compared to the
- 15 *mariner*-hybridising fragments present in chick 13 genomic DNA and found to co-migrate with the approximately 8kb fragment. The pZap13 clone was sequenced using primers near the 5' and 3' end of *mariner*, designed to sequence across the ends of the element into the flanking genomic DNA:

- 20 left-end primer 5'-TCGGCACGAAACTCGACATG
right-end primer 5'-GCAAATACTTAGAATAAATG

Example 3: Analysis of Chick Embryos after injection of *mariner* plasmid

- A series of experiments were carried out in which a plasmid carrying the active
- 25 *mariner* element *Mos1* (Figure 1(a)) (Medhora *et al Genetics* 128 311-318 (1991)) was injected into chick zygotes using established procedures. Purified *mariner* transposase protein was included in approximately half of the injections. A total of 97 zygotes were injected, 51 with plasmid plus transposase protein. DNA was extracted from tissues from embryos that survived for a least 12 days of incubation
- 30 but died before hatch, and from the chorioallantoic membrane of hatched chicks.

These DNA samples were analysed by PCR to detect simultaneously the *mariner* element (MAR) and the plasmid vector (PBS, Figure 1(a)). The copy number of *mariner* and plasmid vector sequences were estimated with respect to the amount of chicken genomic DNA present: single copy (one copy or more per genome equivalent), 0.05-0.5 copies per genome and less than 0.05 copies per genome. 44 of the manipulated embryos survived for at least 12 days of incubation, 23 after injection of p*Mos1* plus transposase protein.

The results of the PCR analysis for the presence of *mariner* and plasmid vector, after injection of p*Mos1* with and without transposase protein, are shown in Table 1. These results are compared graphically to results from injection of a standard gene construct derived from the lysozyme gene (A. Sherman, unpublished results) in Figure 1B. The frequency at which "single-copy" embryos were found after injection of p*Mos1* was dramatically higher than in a lysozyme construct experiment. Less than 1% of the embryos from the lysozyme experiment contained the construct at a level equivalent to one copy per genome in construct to 27% of the embryos injected with p*Mos1*. The results from analysis of embryos injected with or without transposase are very similar (Table 1, Figure 1(b)). This indicates that, if the *mariner* sequences detected in the PCR analysis are present as a result of transposition, then transposition must be able to take place in the absence of exogenous transposase protein.

The embryo samples were analysed for presence of the plasmid vector of p*Mos1*. Of the 29 embryos analysed after injection with p*Mos1*, with or without transposase protein, which contained *mariner* at a level estimated as above 0.05 copies per genome, 6 (21%) also contained plasmid vector sequences (Table 1). The lack of detectable plasmid vector sequences in the remaining embryos in which *mariner* was present, suggested that the *mariner* element in p*Mos1* had transposed out of the plasmid construct, potentially into the chicken genomic DNA.

Example 4: Germline transmission of *mariner*

Three chicks from the above experiment survived to sexual maturity. One chick was identified at hatch as potentially transgenic for *pMos1*. Both *mariner* and plasmid vector sequences were detected by PCR in DNA from chorioallantoic
5 membrane and from blood samples, with a copy number of between 0.1 and 0.5 genome equivalents. This estimate was confirmed when the cockerel reached sexual maturity by analysis of semen samples. This cockerel was crossed with stock hens and hatched offspring screened to detect transgenic chicks. A total of 93 G₁ chicks were screened, 27 (29%) of which were identified by PCR as
10 transgenic for *mariner*.

To determine the frequency of *mariner* insertions in the germline of the G₀ cockerel and the number of different transposition events that had occurred, individual G₁ chicks were analysed. Genomic DNA samples from 23 chicks were
15 analysed by Southern blotting to identify the number and size of restriction fragments that contained insertions of *mariner*. The genomic DNA samples were digested with restriction enzymes BamHI and HindIII, that do not cut within *mariner* itself (Figure 1(a)), and hybridised with a *mariner*-specific probe (Figure 2(a)). Analysis using a further restriction enzyme, EcoRI (Figure 2(b)), that also
20 does not cut within *mariner*, enabled us to determine the number of *mariner*-hybridising restriction fragments present in the different G₁ chicks. Each G₁ chick was classified by the size of BamHI/HindIII and EcoRI, *mariner*-hybridising restriction fragments (Table 2). Six different fragments containing *mariner* were identified and an example of each is shown in Figures 2(a) and (b). Three
25 fragments (Table 2, Figures 2(a) and (b), lane 7), were the most common and were clearly present in the parent cockerel (Figures 2(a) and (b), lane 8). Three of the G₁ chicks were identified by PCR as containing the plasmid vector sequences, as well as *mariner*. Southern transfer analysis (e.g. Figures 2(a) and (b), lane 1) indicated that they contained a 5kb (BamHI/HindIII digest) or an 8kb (EcoRI
30 digest) restriction fragment. This observation indicates that these transgenic birds resulted from integration of multiple copies of the intact *pMos1* plasmid, which

explains the detection by PCR of plasmid vector sequence in genomic DNA samples from the G₀ cockerel. The analysis of genomic DNA from G₁ chicks suggested that *mariner* had transposed from p*Mos1* into the chromosomes of the G₀ cockerel at an early stage of development, and that multiple transposition events
5 had taken place.

Example 5: Transposition of *mariner* into the chicken genome

To demonstrate that the restriction fragments hybridising to *mariner* do correspond to copies of *mariner* integrated into chicken genomic DNA, a restriction fragment
10 containing a single copy of *mariner* was cloned from genomic DNA of one G₁ chick (chick 13; Figure 2(b), lane 2). A library of EcoRI fragments from chick 13 was constructed and screened with a *mariner* probe. A clone containing an 8.2kb fragment, corresponding to the lower band in Figure 2(b) lane 2, was isolated. This clone, pZAP13, was used to reprobe the Southern blot shown in Figure 2(b),
15 (Figure 3(a)). The probe identified a series of EcoRI restriction fragments in all the chicken genomic DNA samples, including DNA from a wild-type chick (Figure 3(a), lane 9). The *mariner* hybridising fragments are also faintly detectable. The clone was also analysed by DNA sequencing, using primers internal to the ends of *mariner*, designed to prime sequence over the ends of the inserted *mariner*
20 element, if complete. The sequence generated (Figure 3(b)) corresponds exactly to the sequence of the ends of the *mariner* element but is flanked by sequences that differ from the *Drosophila* genomic DNA adjacent to the element in p*Mos1*. The element present in the chicken DNA is flanked by TA dinucleotide repeats, the sequence characteristically generated by *mariner* transposase-mediated
25 transposition. These results indicate that *mariner* had integrated into chicken chromosomal DNA by transposition from p*Mos1*.

Example 6: Source of Transposase Function

There was no evidence that the inclusion of transposase protein with the p*Mos1*
30 plasmid in the zygote injection experiments was necessary for transposition (Figure 1(b) and Table 1). The transposase activity could have been due to expression of

the *Mos1* transposase gene or an endogenous activity present in the chick zygote. In order to distinguish between these possibilities, a series of zygote injection experiments was carried out using a construct in which the *mariner* transposase gene was inactivated by insertion of the tetracyclin resistance gene (Tet^R) within the transposase coding region (pMos1Tet). Transposase protein was again included in approximately half of the zygote injections. DNA samples from embryos and chicks were analysed by PCR for the presence of the Tet^R gene and the plasmid vector and their copy number estimated. 29 embryos injected with plasmid alone and 34 with plasmid plus exogenous transposase were analysed and the results are shown graphically in Figure 4. The proportion of embryos containing the Tet^R sequence at a level above 0.05 genome equivalents (17% of embryos without transposase and 24% of embryos with transposase) is much lower than detected after introduction of intact *mariner* (66% of embryos without transposase and 78% of embryos with transposase). All of the embryo DNA samples that contained *mariner* sequences also contained detectable amounts of the plasmid vector (data not shown). The small number of embryos that contained the construct at a single level could have been the result of random integration of the intact plasmid. Again, there was no evidence for function of the exogenous transposase. These results suggest that the transposase activity that lead to transposition of the *mariner* element from pMos1 into the chicken genomic DNA was derived from expression of functional transposase by the construct. The results do not exclude the possibility that the exogenous transposase protein was functional, but they do indicate that it was not necessary for transposition of *mariner* from pMos1.

25

Example 7: Germline Stability of Integrated *Mariner* Elements

Two G_1 birds, cockerels 3 and 7, that each had a single copy of *mariner* integrated at different chromosomal sites (Figures 2(a), lanes 4 and 5) were selected to analyse stability of the elements after germline transmission to the G_2 generation. They were each crossed with stock hens, DNA extracted from resulting embryos and screened by PCR to identify transgenic embryos. The ratios of transgenic to

30

non-transgenic offspring from cockerel 3 (65:59) and cockerel 7 (64:57) did not differ significantly from the expected 1:1 Mendelian ratio. The genomic DNA from transgenic embryos was digested with BamHI and HindIII and the pattern of *mariner*-hybridising fragments compared to the single band present in the transgenic parent. All of the transgenic offspring from both cockerels had a single *mariner* band that co-migrated with the restriction fragment present in the parent cockerel (data not shown). There is no evidence of instability of *mariner* after transposition, although a low level of instability would not have been detected.

10 Example 8: Testing *mariner* transposition in mice

An intact plasmid construct (p*Mos1*), will be injected into the pronucleus or the cytoplasm of mouse fertilised eggs at a concentration of approximately 1.5ng/μl. The method used is as described by Whitelaw *et al* (*Biochemical J.* 286 31-39 (1992)) and is based on the work of Brinster *et al* (*Proc. Nat'l Acad. Sci. USA* 82 4438-4442 (1985)). In some experiments recombinant-derived, purified *mariner* transposase protein or mRNA will be included. The mouse embryos will be transferred to surrogate mothers and new-born mice will be screened to identify any transgenic for *mariner*. All transgenic mice will be analysed further to determine if the *mariner* element is present in the mice as a result of transposase-catalysed transposition or random integration of the whole plasmid construct.

Discussion

The present studies show that the *Drosophila mauritiana* transposable element *mariner* can transpose into the chicken genome after its introduction into the chick zygote by microinjection. The fate of a *mariner*-containing plasmid was analysed after injection into chick embryos at the zygote state and embryo development of at least 12 days. In contrast to the previous results, following the same procedures but using a variety of gene constructs, it was found that *mariner* was present at a level equivalent to one copy per cell in over 20% of embryos. The plasmid vector

that carried the *mariner* element was not detectably present in almost 80% of these embryos. These results suggested that the *mariner* element transposed out of the original plasmid and had integrated into the chicken genome. This interpretation was confirmed by analysis of a cockerel transgenic for *mariner* that survived to sexual maturity and transmitted copies of *mariner* to nearly 30% of his offspring. Analysis of individual G₁ birds showed that a total of six different insertions of *mariner* were present in different individuals. Furthermore, isolation of a single copy of *mariner* from a G₁ transgenic bird confirmed that a complete element had transposed into chicken genomic DNA and that the transposition event had generated the expected TA repeat at the insertion site (Bryan *et al Genetics* 125 103-114 (1990)). No evidence for stability of integrated copies of *mariner* was obtained after germline transmission to the G₂ generation.

The frequency of *mariner* transposition into the chicken genome indicated by this analysis is high (over 20%), although this has to be confirmed by the generation of additional transgenic birds. The frequency of germline transformation obtained by introduction of the same *mariner* element into *Drosophila melanogaster* varied between 4 and 31% (Garza *et al Genetics* 128 303-310 (1991)), a comparable frequency. The proportion of G₁ birds that inherited *mariner* from the G₀ cockerel was approximately 30%, a 10-fold higher transmission frequency than obtained after introduction of linear gene constructs (Love *et al Bio/Technology* 12 60-63 (1994)). The analysis of G₁ birds indicated that there had been multiple insertions of *mariner*. Two possible explanations cannot yet be distinguished between either that several independent transposition events took place from the introduced plasmid or that a copy of *mariner* transposed into the chicken genome and that this was followed by secondary transposition events. The fact that two copies of *mariner* were stably transmitted to the G₂ generation suggests that, once integrated into the chicken genome, *mariner* elements are stable. *Mariner* is also stable after transposition into the *D. melanogaster* genome with an excision rate estimated as below 0.1% (Lohe *et al Genetics* 140 183-192 (1995)).

Purified transposase protein was included with *pMos1* DNA in half of the microinjections but the PCR analysis suggested that the frequency of transposition was not increased by the addition of the enzyme. No transposition was detected when the transposase gene was inactivated by insertion of the Tet^R. It is therefore
5 concluded that the *mariner* transposition events observed were catalysed by expression of the transposase gene in *pMos1*. Previously it has been shown that plasmid DNA injected into the chick zygote is replicated approximately 20-fold during the first 24 hours of embryo development (Sang, H. M., and Perry, M. M., *Mol. Reprod. Dev.* 1 98-106 (1989)) and that expression of a reporter gene
10 construct can be detected within 9 hours of injection (Perry *et al Roux's Arch. Dev. Biol.* 200 312-319 (1991)). It is therefore predicted that there will be a high copy number of *pMos1* per cell during the early stages of development that can act as template for transcription of the transposase gene. Even if the transcription and translation is ineffective sufficient transposase may be synthesised to catalyse
15 transposition. Once the *mariner* elements have integrated they are apparently stable, even though they carry an intact transposase gene. Expression of the transposase gene may be very inefficient once the elements are incorporated in chicken chromosomes and there will only be a small number of copies per cell.

20 These results, and recently described evidence for transposition of *mariner* in zebrafish (Fadool *et al Proc. Nat'l. Acad. Sci. USA* 95 5182-5186 (1998)), are encouraging evidence to support the development of *mariner* as a vector for transgenesis for vertebrates. The use of a *mariner*-derived vector for transgenesis has several potential advantages, particularly for transgenic manipulation of
25 poultry. The frequency of integration may be increased above the level currently possible. The observation that multiple integration events are present in the germline of one G₀ transgenic bird suggests that several transgenic lines, with insertions at different genomic sites, may be established by breeding from one founder bird. There is accumulating evidence that expression of single copy
30 transgenes is less subject to down-regulation of expression than transgenes integrated in multicopy arrays (Garrick *et al Nature Genetics* 18 56-59 (1998)).

The fact that *mariner* vectors will integrate transgenes as single copies may have the additional advantage of resulting in more predictable levels of transgene expression. It is planned to investigate methods to provide the transposase activity *in trans* to a *mariner* vector carrying a transgene. The frequency of transposition of a *mariner* element modified to incorporate a transgene and the size of transgene a *mariner* vector can carry have to be established. Analysis of transgene expression will establish if expression of transgenes introduced in *mariner* vectors as single copies is more predictable than expression of transgenes in multicopy arrays. *Mariner* is only one of the superfamily of *mariner*-like-elements which have potential for development of vectors for transgenesis (Dawson, A. and Finnegan, D. J., *Nature Bio.* 16 20-21 (1998); Raz *et al* *Current Biol.* 8 82-88 (1998)). Future developments will demonstrate if particular elements are more effective as vectors than others or if specific elements are more useful for one application than another.

TABLE I
PCR analysis of DNA from embryos and clicks that developed after
injection of pMosI, with or without addition of recombinant-derived transposase protein

Genome equivalents ^a	0 < 0.05		0.05 - 0.5		1 (+)		TOTAL
	MAR ^a	PBS ^b	MAR	PBS	MAR	PBS	
No transposase	10 (48%)	19 (90%)	6 (28%)	2 (10%)	5 (24%)	0	21
Plus transposase	5 (22%)	19 (83%)	11 (48%)	3 (13%)	7 (30%)	1 (4%)	23
Total	15 (34%)	38 (87%)	17 (39%)	5 (11%)	12 (22%)	1 (2%)	44

* = Estimation of plasmid copy number is explained in Materials and Methods

a = PCR using primers specific sequence

b = PCR using primers specific to plasmid vector sequences

TABLE 2
Estimated size of *mariner*-hybridising restriction fragments
and their frequency in G₁ transgenic birds.

Figure 2(a)/(b)	Restriction fragment size (kb)		Frequency in G ₁
	BamHI	EcoRI	
2	8	8.2	1
2	4	>12	1
3	>12	>12	1
4	1.7	4.7	17
5	5.1	>12	9
6	10.2	6	5

CLAIMS

1. A method for the preparation of a transgenic animal embryo comprising the step of introducing a *mariner*-like element (MLE) containing a transgene into an animal
5 embryo cell, optionally including the step of introduction of exogenous transposase protein, or a DNA or RNA sequence encoding a transposase.
2. A method as claimed in claim 1, in which the animal is an avian species.
- 10 3. A method as claimed in claim 2, in which the animal is a poultry species, for example *Gallus domesticus*.
4. A method as claimed in claim 1, in which the animal is an ungulate species.
- 15 5. A method as claimed in claim 3, in which the animal is a cow or a bull, sheep, goat, water buffalo, camel or pig.
6. A method as claimed in any one of claims 1 to 5, in which the *mariner*-like element is the transposable element *mariner* from *Drosophila mauritiana*.
- 20 7. A method as claimed in any one of claims 1 to 6, in which the *mariner*-like element containing the transgene is introduced into the cell in a construct.
8. A method as claimed in any one of the preceding claims, in which the
25 introduction of the *mariner*-like element (MLE) is by injection of the MLE into the cytoplasm or into the pronucleus of a zygote or the nucleus of an animal embryo cell, by electroporation or using liposomes.
9. A method for the preparation of a transgenic animal embryo comprising the
30 step of introducing a *mariner*-like element (MLE) containing a transgene into an animal

adult cell, optionally including co-introduction of exogenous transposase protein, or a DNA or RNA sequence encoding a transposase.

10. A method for the preparation of a transgenic animal embryo comprising the
5 step of introducing a *mariner*-like element (MLE) containing a transgene into an animal foetal cell, optionally including co-introduction of exogenous transposase protein, or a DNA or RNA sequence encoding a transposase.

11. A method as claimed in any preceding claim, in which the resulting animal
10 embryo is prepared by (i) removing the nucleus of the cell following introduction of the *mariner*-like element containing the transgene and its insertion into the chromosomes, and (ii) subsequently introducing the nucleus into an enucleated oocyte which is allowed to develop into an animal embryo.

15 12. A method for the preparation of an animal, the method comprising the steps of:
(a) preparing an embryo according to any one of the preceding claims;
(b) causing an animal to develop to term from the embryo; and
(c) optionally breeding from the animal so formed.

20 13. A method as claimed in claim 12, in which the animal embryo is further manipulated prior to full development of the embryo.

14. A transgenic animal prepared by a method according to claim 12 or claim 13.

25 15. The use of a *mariner*-like element in the therapy of a disease condition caused by the absence of a gene or the mutation of a gene.

16. The use of a *mariner*-like element in the preparation of an agent for the prophylaxis or treatment of a disease caused by the absence of a gene or the mutation of
30 a gene.

17. A method of treatment of a disease condition caused by the absence of a gene or the mutation of a gene, comprising the step of introducing a *mariner*-like element containing a transgene into an animal cell, optionally including introduction or
5 transposase protein, or a DNA or RNA sequence encoding transposase.

1/4

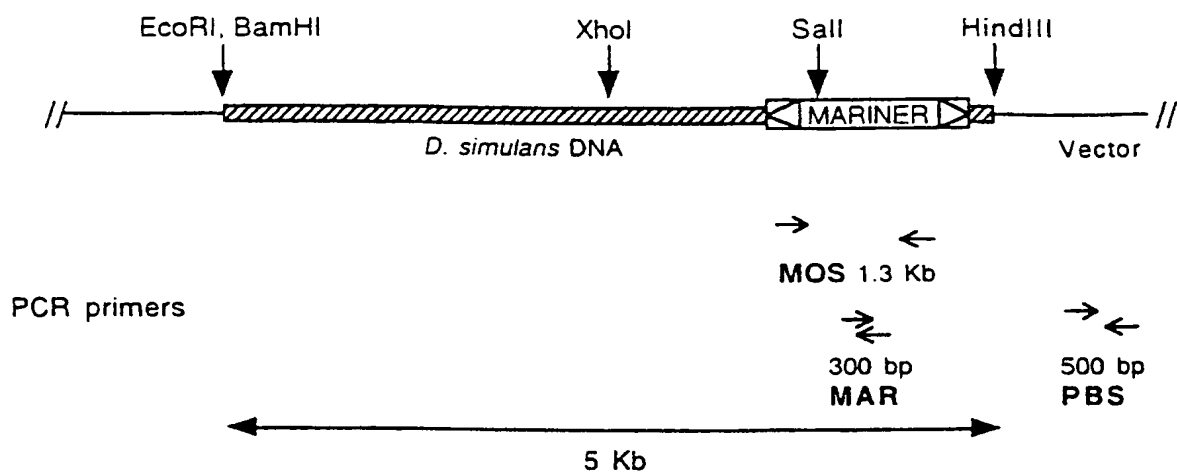


FIG. 1A.

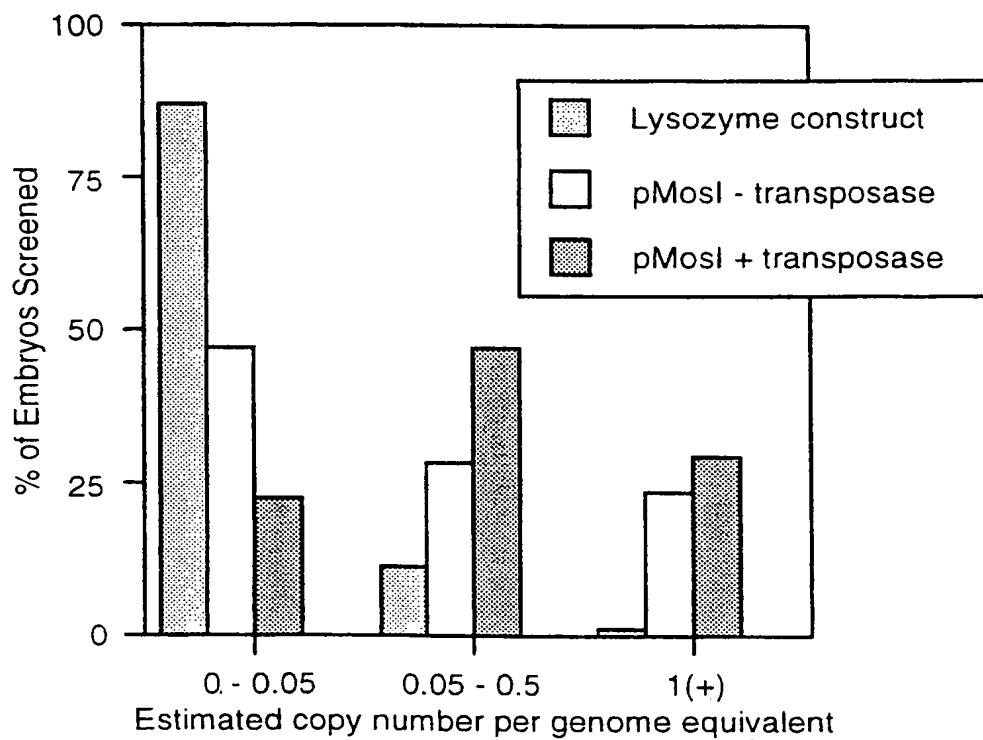
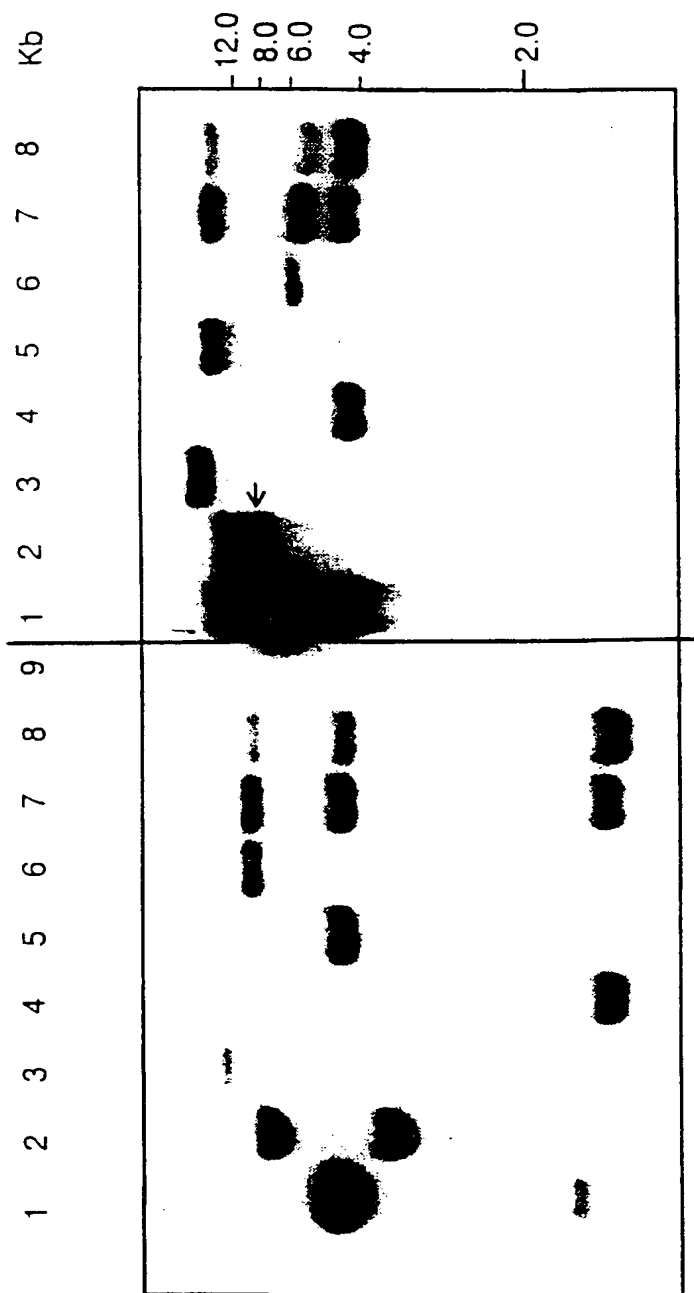


FIG. 1B.

2/4



(b)

FIG. 2

(a)

3/4

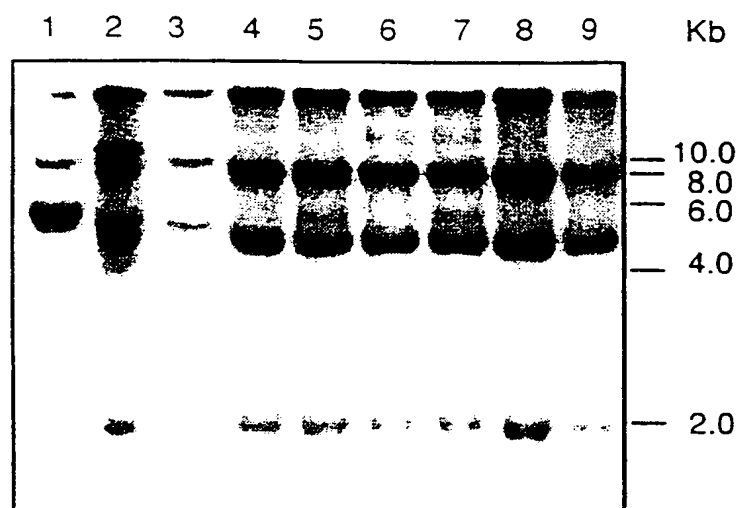


FIG. 3A.

pMos1: 5' TTTAATTTT TTTTCTATAT3'
 TACCAGGTGTAC GTACACCTGATA
pZAP13: 3' CACTTCCTTG TACCAGGTGTAC GTACACCTGATA
 GCTGGGTGTC3'

FIG.3B.

4/4

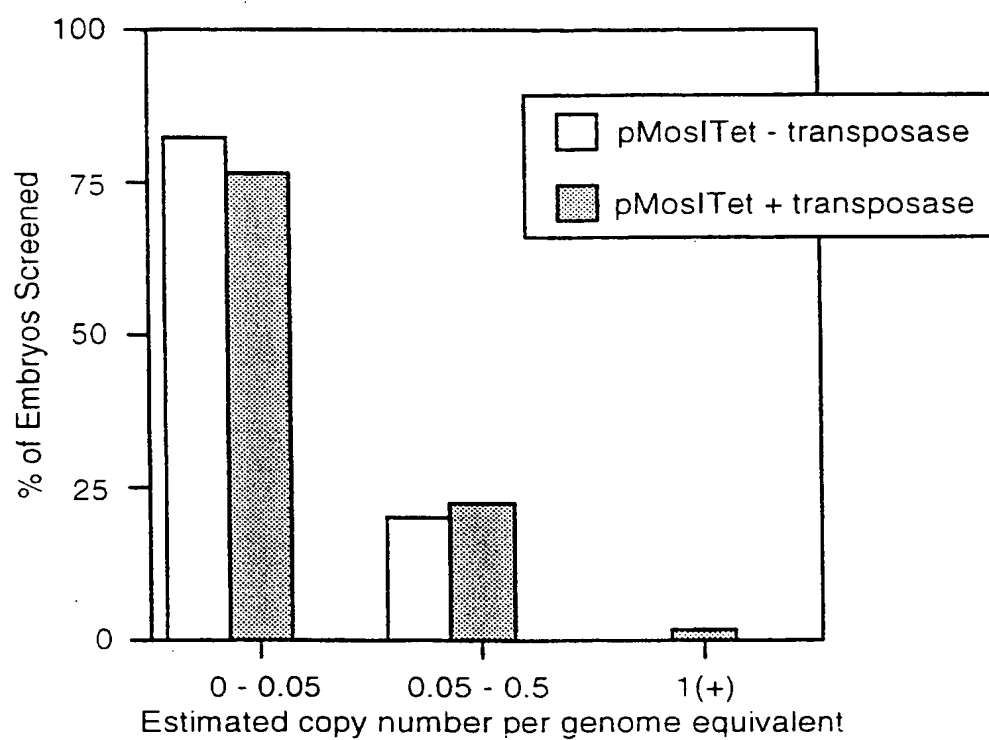


FIG.4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02517

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01K67/027 C12N15/90

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 29202 A (HET NEDERLANDS KANKER INSTITUUT (NL); PLASTERK R.H.A.; VOS J.C.) 14 August 1997	1,6-8, 12-17
Y	see page 2, line 34 - page 6, line 20 see page 24 - page 25; claims	2-5,9-11
Y	WO 97 07669 A (ROSLIN INSTITUTE EDINBURGH (GB); CAMPBELL K.H.S.; WILMUT I.) 6 March 1997 cited in the application see abstract see page 5, line 10-24 see page 7, line 1-11 see page 35 - page 36; claims	2-5,9-11
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

4 December 1998

Date of mailing of the international search report

21/12/1998

Name and mailing address of the ISA

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Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02517

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SMITH I.C. AND FINNEGAN D.: "Mariner transposase" JOURNAL OF CELLULAR BIOCHEMISTRY, vol. Supplement, no. 21A, 10 March 1995 - 4 April 1995, page 227 XP002086553	1,6-8, 12-14
A	Abstract C3-421 see the whole document	6
X	LIDHOLM D.-A. ET AL.: "The transposable element mariner mediates germline transformation in Drosophila melanogaster" GENETICS, vol. 134, 1 July 1993, pages 859-868, XP000576390	1,6-8, 12-14
A	see abstract see page 861, left-hand column, paragraph 1	6
X	VAN LUENEN H.G.A.M. ET AL.: "The mechanism of transposition of Tc3 in C. elegans" CELL, vol. 79, 21 October 1994, pages 293-301, XP002086538	9
A	see page 293, left-hand column, paragraph 1-2 see page 299, right-hand column, paragraph 4	6
P,X	FADDOOL J.M. ET AL.: "Transposition of the mariner element from Drosophila mauritiana in zebrafish" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 95, April 1998, pages 5182-5186, XP002086539 cited in the application see abstract see page 5185	1,6-8, 12-14
A	WO 90 11355 A (NATIONAL RESEARCH DEVELOPMENT CORPORATION (GB); SIMKISS K.) 4 October 1990 see page 1, line 5-12	8
A	PERRY M.M. AND SANG H.M.: "Transgenesis in chickens" TRANSGENIC RESEARCH, vol. 2, no. 3, May 1993, pages 125-133, XP002086865	

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02517

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>SHERMAN A. ET AL.: "Transposition of the Drosophila element mariner into the chicken germ line" NATURE BIOTECHNOLOGY, vol. 16, November 1998, pages 1050-1053, XP002086540</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/02517

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 17
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 98/02517

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